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Antimicrobial susceptibility of *Pseudomonas aeruginosa* isolated from cystic fibrosis patients through Northern Europe

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ABSTRACT

Pseudomonas aeruginosa is a major cause of morbidity and mortality in cystic fibrosis patients. This study compares the antimicrobial susceptibility of 153 *P. aeruginosa* isolates from the United Kingdom (UK) (n=58), Belgium (n=44), and Germany (n=51) collected from 120 patients during routine visits over the 2006-2012 period. MICs were measured by broth microdilution. Genes encoding extended spectrum β-lactamases (ESBL), metallo-β-lactamases and carbapenemases were detected by PCR. Pulsed Field Gel Electrophoresis and Multi-Locus Sequence Typing were performed on isolates resistant to ≥ 3 antibiotic classes among penicillins/cephalosporins, carbapenems, fluoroquinolones, aminoglycosides, polymyxins. Based on EUCAST/CLSI breakpoints, susceptibility was ≤ 30%/≤ 40% (penicillins, ceftazidime, amikacin, ciprofloxacin), 44-48%/48-63% (carbapenems), 72%/72% (tobramycin), and 92%/78% (colistin) independently of patient's age. Sixty percent of strains were multidrug resistant (MDR; European Centre for Disease prevention and Control criteria). Genes encoding ESBL (most prevalent BEL, PER, GES, VEB, CTX-M, TEM, SHV, and OXA), metallo β-lactamases (VIM, IMP, NDM), or carbapenemases (OXA-48, KPC) were not detected. The Liverpool Epidemic Strain (LES) was prevalent in UK isolates only (75% of MDR isolates). Four MDR ST958 isolates were found spread over the three countries. The other MDR clones were evidenced in ≤ 3 isolates and localized in a single country. A new sequence type (ST2254) was discovered in one MDR isolate in Germany. Clonal and non-clonal isolates with different susceptibility profiles were found in 21 patients. Thus, resistance and MDR are highly prevalent in routine isolates from 3 countries, with carbapenem (meropenem), tobramycin and colistin remaining the most active drugs.

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52 **Introduction**

53 Pulmonary infection represents a major cause of morbidity and mortality among cystic
54 fibrosis (CF) patients (1). These patients are therefore regularly exposed to antibiotics for the
55 treatment of infectious exacerbations as well as for the prevention of chronic colonization.
56 *Pseudomonas aeruginosa* is one of the most prevalent bacterial species, especially in the
57 adult population (2). It is well known for its genetic plasticity and capacity to accumulate
58 resistance mechanisms, including acquisition of foreign genetic material (3). The
59 percentage of patients colonized by *P. aeruginosa* has decreased in recent years (2) but,
60 with improved life expectancy, the absolute number of colonized patients has increased. It
61 has also been proposed that multidrug resistant (MDR) strains are more frequent in older
62 patients, primarily due to cumulative exposure to antibiotics (2). A further reason for the
63 spread of antibiotic resistance in CF patients is the dissemination of MDR clones. The
64 Liverpool Epidemic Strain (LES), first described in 1996 (4), has proven particularly
65 successful for acquiring resistance mechanisms over the years (5,6) and for spreading from
66 the UK to other countries such as Canada, Spain and Australia (7).
67 In this study, we compared the antimicrobial susceptibility of *P. aeruginosa* isolated from CF
68 patients in the United Kingdom (UK), where the MDR LES clone is known to be highly
69 prevalent (5), with an equivalent number of strains collected in Germany and Belgium, where
70 no specific survey has been published over the last years. We determined the presence of
71 co-resistance to unrelated antibiotic classes and its possible association with MDR clones.
72 We found that resistance was high in the three countries, but not related to the dissemination
73 of a specific MDR clone in Germany or Belgium. Carbapenems, tobramycin, and colistin
74 remain the most active drugs against *P. aeruginosa* respiratory isolates. Importantly, no
75 carbapenemases were detected in these strains.
76

Materials and methods

Bacterial isolates

A total of 153 clinical *P. aeruginosa* isolates were selected at random among those collected between 2006 and 2012 in 3 CF centers from Belgium (*Hôpital des enfants malades Reine Fabiola/Erasme Hospital*, n = 44); Germany (University Hospital of Münster, n = 51) and UK (Queen's University of Belfast, n = 58) during routine visits. The details on the collection are shown in Table 1. When successive strains were collected from a single patient, only those collected at the first occasion were considered. Nevertheless, more than one isolate were analyzed for some patients based on differences in their phenotypic appearance (see Figure S1 in supplemental material).

Antibiotics

The following antibiotics were obtained as microbiological standards (with abbreviations and potencies shown in parentheses): amikacin disulfate (AMK; 74.80%), colistin sulfate (CST; 79.64%); piperacillin sodium (PIP; 94.20%), and ticarcillin disodium salt (TIC; 85.25%) from Sigma-Aldrich, St. Louis, MO; ciprofloxacin (CIP; 85.00%) from Bayer, Leverkusen, Germany; and tobramycin (TOB; 100%) from Teva, Wilrijk, Belgium. The remaining antibiotics were obtained as the corresponding branded product in Belgium for intravenous use and complying with the provisions of the European Pharmacopoeia with respect to content in active agent: ceftazidime as Glazidim® (CAZ; 88.20%) from GlaxoSmithKline, Genval, Belgium; imipenem as Tienam® [also containing cilastatin which does not have any antibacterial activity] (IPM; 45.60%) from MSD, Brussels, Belgium; meropenem as Meronem® (MEM; 74.00%) from AstraZeneca, Brussels, Belgium; piperacillin-tazobactam as Tazocin® (TZP; 97.00%) from Wyeth, Louvain-La-Neuve, Belgium [now part of Pfizer].

Susceptibility testing

Minimal Inhibitory Concentrations (MIC) were determined by microdilution in cation-adjusted Mueller-Hinton broth following CLSI (Clinical and Laboratory Standards Institute) recommendations, using *P. aeruginosa* ATCC 27853 as quality control strain (8). Susceptibility was assessed according to the interpretive criteria of both the European Committee on Antimicrobial Susceptibility Testing (EUCAST) (9) and the CLSI (8). Isolates were considered as multi-drug resistant (MDR) if resistant to at least three antibiotic classes among those tested (penicillins/cephalosporins, carbapenems, fluoroquinolones, aminoglycosides and polymyxins), according to ECDC (European Centre for Disease Prevention and Control) criteria (10).

Screening for extended-spectrum β-lactamases (ESBL) and carbapenemases

For all isolates (n=51) showing MICs > 8 mg/L for ceftazidime and meropenem, bla_{TEM}, bla_{SHV}, $bla_{CTX\text{-}M}$ (groups 1, 2 and 9), bla_{VIM}, bla_{IMP}, bla_{KPC}, and bla_{NDM} gene families were detected by real-time multiplex PCR, using group-specific primers ([11-13] and references cited therein). Other genes encoding OXA (1,2,9,10,18,20,23,24,30,48, 58,198), BEL (1 to 3), PER (1 to 5, and 7), GES (1 to 18), and VEB (1 to 7) enzymes were also detected by multiplex PCR.

Molecular typing

All MDR isolates in the collection showing co-resistance to penicillins and/or cephalosporins and two other classes (n=56) were characterized by Pulsed-Field Gel Electrophoresis (PFGE) analysis (14). In addition, 42 pairs of isolates collected simultaneously and in the same sample from 21 patients (see Figure S1) but differing in their susceptibility profile to at least one class of antibiotics were also genotyped by PFGE to determine their genetic relatedness. The pulsotype classification criteria designated a pulsotype by one or two letters including patterns showing zero to six DNA fragments differences (14). An epidemic

pulsotype was defined as a pulsotype recovered from ≥ 2 patients while a sporadic pulsotype was recovered only once.

Multilocus sequence typing (MLST) was performed on a representative strain of epidemic pulsotypes detected in ≥ 3 strains, as previously described (15). The reference LES B58 strain (4) was used as control. MLST data were uploaded to the *P. aeruginosa* MLST Database (http://pubmlst.org/paeruginosa) for allele type and sequence type (ST) assignments (16).

Results

MIC distributions

Table 2 shows the MIC distribution for 9 antipseudomonal drugs against 153 isolates collected from 120 CF patients originating from three different countries over the 2006-2012 period, together with the percentage susceptible and resistant based on both EUCAST and CLSI interpretive criteria. The corresponding MIC cumulative distributions are illustrated in Figure S2. Resistance was high in this collection. Using the EUCAST or the CLSI "R" breakpoints, respectively, full resistant isolates were ≥71% or ≥54% for penicillins (ticarcillin, piperacillin, piperacillin-tazobactam), 69% or 59% for ceftazidime, 61% or 46% for amikacin, 56% or 27% for ciprofloxacin, ≥20% for carbapenems, and 28 or 16% for tobramycin. Full resistance to colistin was noted for only 8% of the isolates. Strains resistant to ceftazidime and meropenem were screened for the expression of frequent ESBLs, metallo β-lactamases, and carbapenemases, which returned negative results.

Cross- or co-resistance

Cross- or co-resistance was examined among pairs of antibiotics. Cross-resistance is defined as a single resistance mechanism that confers resistance to antimicrobial molecules with a similar mechanism(s) of action. It thus describes resistance to an entire class of antibiotics, or to different classes of agents with overlapping drug targets, or to different classes of antibiotics that are substrates for the same broad-spectrum efflux system. Co-resistance rather refers to the presence of different mechanisms of resistance in the same bacterial isolate, and is thus necessarily confers resistance to unrelated antibiotic classes (17). Ninety-four strains were considered as MDR according the ECDC (10). The right upper part of Table 3 shows the percentage of strains showing cross- or co-resistance to pairs of antibiotics according to EUCAST criteria. About 2/3 of the strains were resistant to both penicillins and ceftazidime and more than 40%, to penicillins and ceftazidime together with amikacin or ciprofloxacin. Co-resistance between any studied drug and tobramycin,

meropenem, and colistin was lower than 28%, 20% and 8%, respectively. Of note, only 4 strains in the whole collection were co-resistant to meropenem, tobramycin, and colistin (Figure S3).

The left lower part of Table 3 shows the correlation coefficient between the individual MIC for each pair of antibiotics, with the corresponding multivariate analysis presented in details as supplementary Figure S4. The highest degrees of correlation (> 0.75) between individual MICs were observed for ticarcillin *vs*. ceftazidime, piperacillin *vs*. piperacillin-tazobactam, ceftazidime *vs*. piperacillin-(tazobactam), imipenem *vs*. meropenem, and amikacin *vs*. tobramycin, suggesting common mechanisms of resistance between these pairs of antibiotics. Yet, differences in the intrinsic potency were nevertheless observed between these pairs of drugs throughout the collection; they are illustrated in Figure S4 and associated Table B: tazobactam reduced the MIC of piperacillin by a factor of 1.5 dilution, while ceftazidime MICs were 0.5 and 1 dilution lower than those of ticarcillin and piperacillin respectively, and similar to those of piperacillin-tazobactam. Meropenem MICs were 1 dilution lower than those of imipenem, and tobramycin MICs were 3 dilutions lower than those of amikacin.

Typing of MDR isolates

Among the 94 MDR isolates, most were resistant to penicillins and/or cephalosporins. Only those showing resistance to at least 2 other classes (n = 56) were characterized by PFGE analysis. A high genetic diversity was observed, with 19 sporadic pulsotypes and 9 epidemic pulsotypes (Table 4). With the exception of pulsotype YY recovered for 1 or 2 isolates in the three countries, each epidemic pulsotype remained localized in a single country. The CA epidemic pulsotype found in 3/4 of the UK isolates corresponded to the pulsotype of the LES clone. MLST analysis of epidemic pulsotypes CA, H and YY showed ST146, ST2254 (new ST) and ST958, respectively (data not shown).

PFGE analysis was also performed on 42 isolates collected as pairs from 21 patients and displaying different susceptibility profiles (Table S1). In twelve patients, the pair of *P. aeruginosa* isolates had the same pulsotype, while the 9 other patients had isolates with different pulsotypes.

Analysis per country or age group

Because of the genetic diversity observed between countries, we then examined the distribution of susceptible, intermediate (when applicable) and resistant isolates classified based on the country where they were collected (Figure 1). Susceptibility rates differed among countries, with lower resistance in Belgium (significant for all antibiotics except ticarcillin and ciprofloxacin) and higher resistance in Germany and UK (significant for piperacillin-tazobactam in Germany and for imipenem, ciprofloxacin, and colistin in UK) as compared to the mean value for the whole collection. There was no significant correlation between the patient's age when the isolate was collected and the number of antibiotic classes to which the isolate was resistant (Figure S5).

206 **Discussion**

207 In this study, we examined antibiotic susceptibility of a collection of *P. aeruginosa* isolated
208 from CF patients in three Northern European countries during routine examination, which
209 provides a broader view than the majority of previous surveys that have focused on a single
210 country (18-20) or a single center (21-23). A key observation is that resistance rates were
211 high in this population, confirming previous studies with CF patients (2), and notably
212 A.9.much higher than that which has been reported for isolates collected in Northern
213 European from intensive care units (24-26). Resistance rates were also higher than those
214 previously reported for strains from CF patients in a German survey from the University of
215 Würzburg except for tobramycin (27; collection in 2006), or in a multicentric study in the UK,
216 except for meropenem and ciprofloxacin (28; collection in 2000). Moreover, a high degree of
217 cross- or co-resistance among antibiotics was observed, which is important from both a
218 pharmacological and clinical perspective.
219
220 From a pharmacological perspective, we noticed, as expected, significant correlations
221 between MIC values for antibiotics belonging to the same or similar classes (penicillins and
222 ceftazidime or other penicillins, imipenem and meropenem, and amikacin and tobramycin),
223 but with systematic differences in the potency of each antibiotic within these pairs (see
224 Figure S3 and related Table B). Focusing on β-lactams, the impact of tazobactam on
225 piperacillin activity was modest, but of the same order of magnitude as that observed on MIC
226 distribution for wild-type strains reported by EUCAST (29), probably denoting the inhibition
227 by tazobactam of the low basal levels of AmpC produced by the wild-type strains (30,31).
228 Likewise a higher potency of ceftazidime compared to penicillins and of meropenem
229 compared to imipenem is reported in wild-type EUCAST distributions (29). Thus differences
230 in potency among these pairs of drugs in our collection are likely to reflect differences in
231 intrinsic activity rather than in vulnerability to resistance mechanisms. Remarkably no
232 carbapenemase production was apparent in this collection. A same finding was reported in

two recent reports studying *P. aeruginosa* collected over the same period of time as those examined here. The first of these studies was performed in Australia and examined successively a collection of 662 carbapenem-resistant isolates assembled in 2007-2009 from diverse CF centers and of 517 isolates collected in a single CF center in 2011 (32). The second study was performed in Brazil and analyzed isolates from 75 patients collected in 2010-2011 (19). To the opposite, carbapenemases have been detected in 63 out of 217 *P. aeruginosa* collected from CF patients in China (22). The prevalence of carbapenemase genes could, however, be different in other bacteria infecting CF patients, but there is no large survey published so far in other Gram-negative species (33,34).

Thus, carbapenem resistance in CF European isolates is probably primarily mediated by the combined effect of AmpC and of a reduced accumulation (porin mutations and/or increased efflux) (35; Chalhoub *et al,* submitted for publication]. Of note, however, carbapenem resistance has previously been described in the LES clone (5) but the underlying mechanism(s) have not been investigated to date. For aminoglycosides, the higher potency of tobramycin over amikacin in our collection also reflects what is observed in MIC distributions of wild-type strains assembled by EUCAST (29). Tobramycin has been described as a poorer substrate than amikacin for the efflux pump MexXY-OprM considered as responsible for natural and adaptative resistance to aminoglycosides in *P. aeruginosa* (36,37).

Considering our findings from a clinical perspective, a high degree of cross-resistance was observed between penicillins and ceftazidime, which was expected. However, a high degree of co-resistance was also apparent between these antibiotics and both ciprofloxacin and amikacin, resulting in 60 % of the isolates being categorized as multidrug resistant. In contrast, meropenem, and colistin, and to a lesser extent, tobramycin, were active against a large fraction of the isolates with few strains co-resistant to these three antibiotics. Tobramycin and colistin by inhalation are often considered as first line for the eradication of early *P. aeruginosa* infection and tobramycin, also for chronic therapies (38-40). High

concentrations delivered by this route of administration may help to overcome resistance (41,42).

We also noticed an important genetic diversity among multi-resistant isolates collected in Belgium and Germany while those collected in the UK belong in majority to the Liverpool Epidemic Strain (LES) clone. Global studies of *P. aeruginosa* population structure concluded that CF isolates present a high genetic diversity but nevertheless belong to a 'core lineage' ubiquitous in the natural environment (43), which is highly suggestive of a direct colonization of the patients from the environment. However, a series of epidemic clones have been described (7) among which the LES (4) representing 18 of the 24 MDR isolates collected in the UK in our study, and the ST17 (7), which differs by only 1 nucleotide from the ST958 found in the three countries investigated here. The new ST2254 we described was distinct from ST146 (LES clone, 5 alleles difference) and ST958 or ST17 (6 alleles difference).

We observed that a single patient can be colonized by different strains and, conversely, that clonally-related strains isolated at the same time from a single patient can harbor diverse susceptibility profiles. This could be a consequence of the previously described phenotypic variability among isolates with the same colony morphotype and being part of a single clonal lineage (44,45), as well as of recombination occurring *in vivo* and generating phenotypic and genetic diversification (46,47).

Although limited, differences in resistance rates between Belgium and the other two other countries are raising questions about segmentation of clone distribution. For strains collected in the UK, higher resistance is clearly related to the high prevalence of the LES clone, which has been described as exhibiting a large proportion of MDR isolates (5). Of interest, we observed different resistance profiles within this clone, which is coherent with the previously described phenotypic variability among LES isolates (6). The ST958 represented in the three countries is also found among the MDR clonal complexes (7). In the German

collection, higher resistance is essentially related to the presence of more sporadic MDR clones than in the two other countries. We cannot exclude differences in therapeutic management of patients among these three centers that may influence resistance selection (48) but this specific aspect was not within the scope of our study.

Resistance rates were not higher in the older population than in children/young adults. The interpretation of these data need to be cautious because (a) we did not follow the evolution of susceptibility over time in single patients and (b) we do not know the age of first colonization for each patient. With this limitation in mind, the fact that MDR isolates could be found in young people and susceptible isolates in adults may suggest that resistance depends on the initial susceptibility of the infecting strain. A link between emergence of resistance and early antibiotic use in CF patients is still controversial, even though underlined in the last report of the Cystic Fibrosis Foundation (2). A recent study in Australia showed that multiresistance in children is correlated with duration of intravenous antibiotic treatment, which was not the case for adults (18). A correlation with antibiotic usage irrespective of patient's age (49) or with time after colonization (6) has also been proposed. In contrast, other studies following the evolution of antibiotic susceptibility in successive isogenic isolates from a single patient suggest either that resistance can occur sporadically (50) or without correlation with the time of isolation (51). In these cases, the presence of mutator variants seems to predetermine the risk of developing resistance over time (6).

Our study has a number of limitations, primarily linked to the fact that samples collected during periodic routine examinations may not correspond to the first isolates of *P. aeruginosa* infections in these patients. Moreover, as we did not have the history of antibiotic use in these patients, we could not determine if there was a potential link between antibiotic usage and subsequent development of resistance. Nevertheless, this collection reflects the situation CF clinicians face daily, where they have to select antibiotics based on susceptibility testing performed on current isolates. In this context, our data may lead to

three clinically-meaningful conclusions. First, susceptibility testing is important to perform even in newly infected patients, because they can be colonized very early by MDR clones. Second, these tests should be performed on more than one colony (especially if different phenotypes are evidenced on culture plates), because of potential population heterogeneity with respect to susceptibility profiles (52). Third, prudent use of highly active drugs should be promoted in order to preserve their efficacy. This implies the use of optimized doses if administered by conventional routes or administration by inhalation to insure high local concentrations that could minimize the risk of selection of resistance.

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533 **Table 1: *P. aeruginosa* collection (2006-2012)**

Country	Number of isolates	Number of patients	Period of sampling
Belgium	44	38	2010
Germany	51	36	2012
United Kingdom	58	46	2006-2009
Total	153	120	

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537 **Table 2: MIC distributions for antipseudomonal antibiotics and corresponding percentage of susceptibility according to EUCAST or**
538 **CLSI breakpoints**

Antibiotic	MIC distribution (mg/L)				Susceptibility according to					
					EUCAST [a]			CLSI [b]		
	min	max	MIC_{50}	MIC_{90}	% S	% I	%R	% S	%I	%R
Ticarcillin (TIC)	1	>512	128	>512	16	NA	84	16	23	61
Piperacillin (PIP)	0.5	>512	256	>512	24	NA	76	24	15	61
Piperacillin-tazobactam (TZP)	0.5	>512	128	512	29	NA	71	29	17	54
Ceftazidime (CAZ)	1	>512	64	512	31	NA	69	31	10	59
Imipenem (IPM)	0.25	128	4	32	48	19	33	48	19	33
Meropenem (MEM)	0.032	256	2	16	44	36	20	63	17	20
Amikacin (AMK)	1	>512	32	128	22	17	61	39	15	46
Tobramycin (TOB)	0.064	>512	2	16	72	NA	28	72	12	16
Ciprofloxacin (CIP)	0.064	64	1	8	24	20	56	44	29	27
Colistin (CST)	0.25	>512	1	4	92	NA	8	78	14	8

[a] EUCAST breakpoints (NA: not applicable [no I category]): **TIC** S≤16 R>16; **PIP** S≤16 R>16; **TZP** S≤16 R>16; **CAZ** S≤8 R>8; **IPM** S≤4 R>8; **MEM** S≤ 2 R>8; **AMK** S≤ 8 R>16; **TOB** S≤ 4 R>4; **CIP** S≤ 0.5 R>1; **CST** S≤ 4 R>4.

[b] CLSI breakpoints: **TIC** S≤16, I=32-64, R≥128; **PIP** S≤16, I=32-64, R≥128; **TZP** S≤16, I=32-64, R≥128; **CAZ** S≤8, I=16, R≥32; **IPM** S≤4, I=8, R≥16; **MEM** S≤4, I=8, R≥16; **CIP** S≤1, I=2, R≥4; **AMK** S≤16, I=32, R≥64; **TOB** S≤4, I=8, R≥16; **CST** S≤2, I=4, R≥8.

S: susceptible; I: intermediate; R: resistant

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Table 3: Percentage of co-resistance among pairs of antibiotics and multivariate correlation between MIC values of each pair of antibiotics for individual strains.

Above the diagonal, figures correspond to the percentage of isolates categorized as resistant to the two antibiotics (row/column) using EUCAST breakpoints. Values highlighted in bold indicate combinations for which resistance is higher than 30 %.

The numbers below the diagonal correspond to the correlation coefficient between individual MIC values for each pairs of antibiotics. Values higher than 0.75 are highlighted in bold characters. See Table 2 for abbreviations of antibiotics and Figure S4 for the details of this analysis.

Percentage of cross- or co-resistance

r value (multivariate correlation)

TIC	**68**	**71**	**69**	31	20	**54**	25	**48**	8
0.78	**CAZ**	**68**	**65**	29	20	**48**	24	**42**	7
0.72	**0.88**	**PIP**	**71**	31	20	**52**	24	**45**	7
0.73	**0.86**	**0.94**	**TZP**	30	20	**50**	24	**42**	7
0.53	0.47	0.47	0.45	**IPM**	16	24	12	24	4
0.66	0.55	0.48	0.54	**0.80**	**MEM**	14	7	18	3
0.37	0.46	0.40	0.36	0.34	0.26	**AMK**	28	**38**	8
0.26	0.40	0.31	0.28	0.29	0.17	**0.90**	**TOB**	22	5
0.26	0.30	0.27	0.28	0.39	0.43	0.31	0.31	**CIP**	6
0.18	0.16	0.14	0.11	0.13	0.04	0.32	0.34	0.01	**CST**

550 **Table 4: Distribution of pulsotypes among the MDR *P. aeruginosa* clinical isolates**

Country	Number of MDR strains	Number of pulsotypes		Number of strains in epidemic pulsotype								
		Sporadic	Epidemic	CA[a]	CK	CM	CD	H	WW	YI	CJ	YY
Belgium	10	3	4	0	0	2	2	0	2	0	0	1
Germany	22	11	5	0	2	0	0	3	0	2	2	2
United Kingdom	24	5	2	18	0	0	0	0	0	0	0	1

551 [a] CA pulsotype corresponds to the LES epidemic clone pulsotype

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553 **Figure 1:** Comparison of the percentage of antibiotic resistance in the collection based on
554 the country of origin of the strain (Belgium (BE): n=44; Germany (DE): n=51; United
555 Kingdom (UK): n=58). Statistical analysis: Chi Square test (p values indicated after the
556 name of the antibiotic); Analysis of means of proportions with α level of 0.05: * denotes a
557 value below the mean and #, above the mean.